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Seasonal and spatial variations in biomarker baseline levels within Arctic populations of mussels (*Mytilus* spp.)

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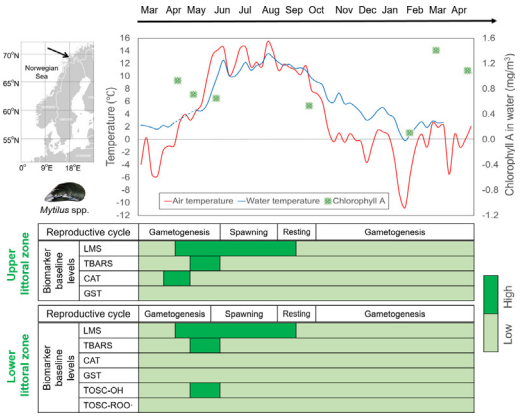
HIGHLIGHTS

- Maturity phase and tidal zone had the strongest influence on biomarker baseline levels.
- Spawning was partial during summer when water temperature ranged from 10 to 14 °C.
- Contaminant levels accumulated in mussels significantly increased from 2010 to 2014.
- Neutral red retention assay has to be adjusted to mussel ambient temperature.

GRAPHICAL ABSTRACT



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ABSTRACT

Climate change and pollution resulting from human activities in the Arctic require reliable monitoring systems in sentinel species. *Mytilus* spp. are used as sentinel species all around the world. The use of *Mytilus* spp. in environmental monitoring requires knowledge about natural variations in pollution biomarkers. Seasonal variations in baseline levels of biomarkers were studied over a year in the mussels from both upper and lower littoral zones in Rakkfjorden, Norway, as they underwent their annual reproductive cycle. Spatial variations of these biomarker baseline levels were measured in five mussel populations within a 60-km radius from Rakkfjorden to investigate universality of the results from the specific population of Rakkfjorden at a regional scale. Seasonal variations in biomarker baseline levels were revealed and seemed to be related to the reproductive state of the mussels and the tidal zone. The mussels appeared to be more sensitive to oxidative stress during gametogenesis in autumn and winter, when having lower lysosome membrane stability and lower baseline levels of antioxidant biomarkers. An increase in baseline levels of these biomarkers was reported during spawning in spring, however, it was not possible to reveal whether these changes were due to spawning, or to a higher metabolic activity in mussels in response to elevated water temperature and food abundance. The differences between the tidal zones were reflected in reduced size of the mussels from the upper littoral zone, their late spawning in the season

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and increased baseline levels of antioxidant biomarkers during the coldest month, indicating a more challenging environment in the upper littoral zone. The spatial study indicated that the biomarker baseline levels measured in Rakkfjorden were no different from the levels measured in the mussels from five other sites and thus, are representative for mussels on a regional scale.

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1. Introduction

Bivalves, such as mussels (family *Mytilidae*), are usually used as sentinel species in environmental monitoring (Caza et al., 2016; Azizi et al., 2018; Breitwieser et al., 2018). They are filter feeders that may absorb and accumulate pollutants in their tissues. They are sessile, so the pollutants bioaccumulated are representative of a specific location. Mussels (*Mytilus* spp.) are used as sentinel species in many parts of the world, both in large environmental monitoring programmes, such as the Mussel Watch Programme in the USA (Goldberg, 1986) and at more local scales (Laffon et al., 2006). For example, in Norway, the blue mussel (*Mytilus edulis*) is used to monitor effects of offshore petroleum activities (Hylland et al., 2008; Brooks et al., 2011).

Increases in oil and gas activities (AMAP, 2010; Harsem et al., 2011), shipping (Smith and Stephenson, 2013) and tourism (Arneberg et al., 2009; Dybedal et al., 2015) in northern regions create increased risks for acute and chronic pollution. Arctic marine environments, especially coastal habitats, are considered to be sensitive areas with high biodiversity, unique ecology and economical value. Biomarkers are commonly used as early warning tools in environmental monitoring (Cajaraville et al., 2000), allowing one to detect alterations at various levels of biological organization in response to pollutants and other possible environmental stressors. Prior to adopting biomarkers for environmental monitoring it is important to have knowledge about their baseline and natural variations (van der Oost et al., 2003), which can be affected by seasonal variations related to cycles of feeding, growth and reproduction (Gonzalez-Fernandez et al., 2016). Also, the biomarker baseline levels can vary due to spatial differences related to local variations in the environmental conditions including within a mussel bed between lower and upper littoral zone (Petes et al., 2007), and on a larger spatial scale, variations in oceanographic regime (Pfeifer et al., 2005; Kamel et al., 2014; Vidal-Liñán, 2015) and pollution (Gagne et al., 2008).

In the present study, baseline levels of biomarkers of oxidative stress (lipid peroxidation, catalase [CAT] activity, glutathione S-transferase [GST] activity, total oxyradical scavenging capacity [TOSC]), and lysosome membrane stability (LMS) were studied. These biomarkers are used in most monitoring programmes and reported in the International Council for the Exploration of the Sea (ICES) guidelines (ICES, 2010). The main objective was to investigate seasonal changes and spatial differences in these biomarkers in mussels *Mytilus* spp. from Arctic coastal sites and discuss them in relation to exogenous (season, water temperature, tidal zone, phytoplankton abundance, pollutants) and endogenous (size, gender, reproductive stage) factors. In the seasonal study, the baseline variations of biomarkers were studied over a year in mussels from upper and lower littoral zones in Rakkfjorden, Kvalsundet, Troms County, Norway (69°49′N 18°55′E, Fig. 1). We hypothesized that the baseline levels of biomarkers vary over the course of a year due to physiological changes associated with the annual reproductive cycle (Schmidt et al., 2013; Gonzalez-Fernandez et al., 2016) and seasonality in environmental conditions (photoperiod, temperature, salinity and food supply) (Bebianno et al., 2007; Brenner et al., 2014). Moreover, differences in biomarkers related to the location of the mussels within the mussel bed were expected (Izagirre et al., 2008; Letendre et al., 2009; Schmidt et al., 2012; Brenner et al., 2014). Exposure to harsher environmental conditions (e.g. desiccation, light, and temperature) for longer periods of time at low tide in the mussels on the upper littoral zone may result in an energy allocation that differs from that seen in mussels from the lower littoral zone (Petes et al., 2008; Tagliarolo et al., 2012).

The aim of the spatial study was to investigate the universality of the results from the seasonal study by examining the same biomarkers in additional mussel populations from the lower littoral zone located within a 60-km radius from the site at Rakkfjorden (Fig. 1). We hypothesized that there would be no differences in biomarkers among populations because the mussels were collected within a short time period (4 days), they were expected to be at the same stage of reproductive development, the chosen sites were generally similar to each other in terms of physical characteristics (rocky shore and sandy bottom), and environmental conditions were relatively pristine, with little background pollution.

The study is unique in its design as it completes the study from the same location performed by Nahrgang et al. (2013) with continuous logging of environmental conditions over the entire year, description of the reproductive cycle of the local mussels, their monthly biomarker baseline levels, and the validation of the findings of this location across a spatial sampling. In addition, measurement of the biomarker baseline levels in the mussels from both upper and lower littoral zones underlines the importance of the tidal zone for future biomonitoring studies previously reported only in few papers (Izagirre et al., 2008; Letendre et al., 2009; Schmidt et al., 2012).

2. Materials and methods

2.1. Environmental conditions

Environmental conditions at the mussel sampling site in Rakkfjorden were measured from 16th February 2013 to 18th April 2014 using air and underwater HOBO Pendant® Temperature/Light 64K Data loggers UA-002-64 deployed above the mussel bed and in the lower littoral zone. Light intensity, air temperature and water temperature were recorded every 5 min and data were then used to calculate weekly means (n = 2016 data points per week). Salinity was recorded every 10 min in the lower littoral zone using a HOBO Conductivity/Salinity Data Logger U24-002-C. Water temperature and salinity were measured once at each site in the spatial study using the YSI Professional 1020 instrument (Pro1020).

Tidal information was obtained from the Norwegian Mapping Authority (http://kartverket.no/en/sehavniva/) to determine the amount of time mussels were air exposed during one tidal cycle (6 h) in the upper and lower littoral zone.

2.2. Determination of total chlorophyll A concentration in seawater

For chlorophyll A (Chl A) quantification, in both seasonal and spatial studies (Table S1), one litre of water was sampled from 20 cm depth in a green glass bottle, and then held cool and dark for a few hours before being filtered. Three subsamples (300 ml) were vacuum-filtered on Whatman GF/F filters (>0.7 μm), and Chl A was subsequently extracted in 5 ml methanol overnight at 4 °C (Holm-Hansen and Riemann, 1978). The next day the concentration of Chl A (mg/m^3) was measured using a Turner Design AU-10 fluorometer calibrated with pure Chl A (Sigma, C6144).

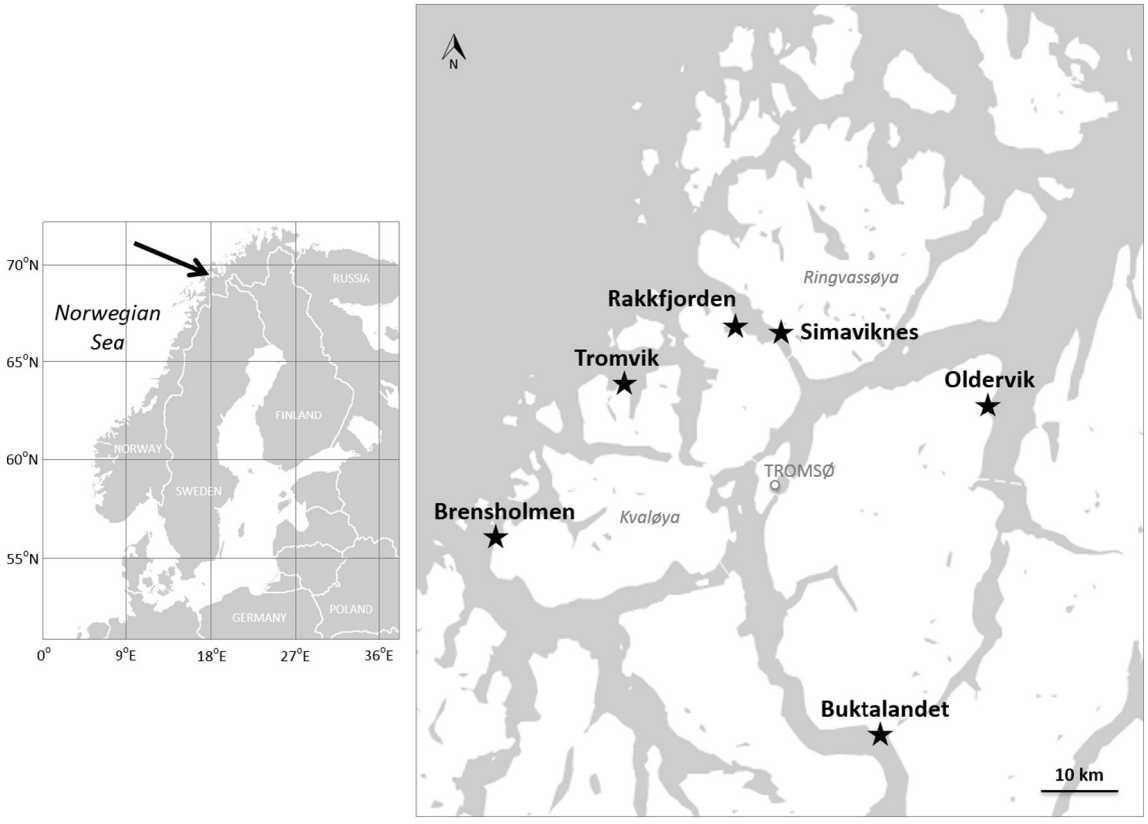

Fig. 1. Sampling sites for the seasonal (Rakkfjorden) and spatial (Simaviknes, Rakkfjorden, Tromvik, Brensholmen, Oldervik and Buktalandet) studies.

2.3. Mussel collection

Mytilus edulis is the dominant mussel species in Rakkfjorden, but *M. galloprovincialis* and some hybrids are also present (Mathiesen et al., 2016). It is difficult to distinguish between the two species and their hybrids by visual inspection of their external shells, so the designation *Mytilus* spp. will be used in this paper.

For the seasonal study, mussels (*Mytilus* spp.) were collected at 50 cm (upper littoral zone) and 80 cm (lower littoral zone) below mean sea level, being out of water for 3.0 ± 0.1 and 1.3 ± 0.1 h per tidal cycle, respectively, each month from 9th April 2013 to 16th April 2014 (Table S1). At each sampling point the largest mussels were selected (n = 9–12 per month from each tidal zone). Mussels were collected by hand during low tide and transported directly to the laboratory for dissection. Mussels from the lower littoral zone were transported in a cool box containing seawater, while the mussels from the upper littoral zone were transported in a cool box that contained paper towels soaked in seawater to provide a cool and moist environment. All mussels were used for histology and biomarker analyses (Table S1).

For the spatial study, mussels (*Mytilus* spp.; n = 30 per site, except n = 39 in Rakkfjorden) were collected from the lower littoral zone of six sites (Fig. 1; Table S1) from 25th July to 28th July 2014 with 12 or 24 h between collection. The mussels were collected by hand during low tide and transported in a cool box containing seawater to the laboratory for dissection. Fifteen mussels from each site were used for histology and biomarker analyses (Table S1), and three pools of 5–8 mussels from each site were prepared to determine the concentrations of polycyclic aromatic hydrocarbons (PAHs) and metals in their soft tissues (Table S1) to evaluate the degree of pollution.

2.4. Dissection

In mussels collected for the seasonal study (n = 9–12 per month from each tidal zone), approximately 0.1 ml of haemolymph was removed from the posterior adductor muscle with a syringe containing approximately 0.1 ml physiological saline (pH 7.3). Collected haemocytes were used to measure LMS, a cellular biomarker for general organism health that assesses the changes in lysosome membrane integrity as a response to contamination, using the neutral red retention assay (Martínez-Gómez et al., 2015). The haemolymph/saline solution was gently mixed and placed in a microcentrifuge tube, from which a 40 μl sample was pipetted onto the centre of a microscope slide. The slide was left in a dark chamber for 15 min to allow adhesion of the cells to the slide. The chamber contained crushed ice and paper towels soaked in water on top of it to provide a cool and humid atmosphere. The average air temperature inside the chamber stayed at 17.7 ± 0.3 °C throughout the whole assay, as it was recommended in Martínez-Gómez et al. (2015), and was likely stable over the year. Following incubation, excess liquid was removed and 40 μl of neutral red solution was added. The neutral red solution was taken up by the haemocyte lysosomes. The ability of the lysosome to retain the neutral red solution was examined by inspection every 15 min for 1 h and then every 30 min for 2 h at 40× magnification using a Leica DM2000 LED light microscope. The test was terminated and the time recorded when over 50% of the haemocyte lysosomes had leaked the neutral red dye into the cytosol.

Shell length, width and height of all mussels (±0.01 mm) were measured using a digital caliper. Length was defined as maximum anterior-posterior distance, width was measured from top hinge joint to lower shell edge distance, and height was measured dorsoventrally at the thickest part of the mussel (Fig. S1). Shell measurements were used to calculate the volume (V) of the mussels as a simplified proxy of the size (Versteegh, 2012):

$$V = 0.455 \times \text{length} \times \text{width} \times \text{height}.$$

Total wet weight (total soft tissues + shell) and shell weight (± 0.0001 g) were recorded using a Sartorius Extend ED124S analytical

lab balance, and the total soft tissue wet weight was computed as total wet weight minus shell weight. Condition index (CI) was then calculated (Damiens et al., 2007):

$$CI = 100 \times \frac{\text{soft tissue wet weight}}{\text{total wet weight}}.$$

Gonads were dissected out and fixed in 4% formaldehyde for histological analysis. Digestive glands were dissected out, weighed (±0.0001 g), snap frozen in liquid nitrogen and stored at −80 °C until analysed for biomarkers (Table S1).

2.5. Histological analysis

Gender and reproductive stage were determined using histological examination of gonads (Table S1). Histological techniques were as described by Bancroft and Stevens (1982). Briefly, the gonadal tissue was dehydrated in ethanol, cleaned in histoclear and then embedded in paraffin wax. Thin sections (5 μm; n = 5–10 per mussel) were prepared, stained with hematoxylin and eosin, examined at 10× magnification by light microscopy, and photographed with a Pixera Pro 150ES camera.

Reproductive stage was assigned to one of four stages (Maximovich, 1985). Stage 0 was the period of gonadal restoration after spawning, when the gonad was filled with connective tissue and a few unreleased gametes (ova/spermatozoa) (Fig. S2A and S2B). Stage I was designated early gametogenesis when follicular walls started to develop and immature gametes (spermatogonia/oogonia) were formed (Fig. S2C and S2D). Stage II was considered to indicate active gametogenesis when the follicules increased in size and contained developing gametes; the follicules occupied about 50% of the gonadal section (Fig. S2E and S2F). Finally, stage III was designated as maturity, when follicules were of maximum size, filled with mature gametes and inter follicular connective tissue was not visible (Fig. S2G and S2H).

2.6. Contaminant burden in soft tissues of the mussels collected for the spatial study

Determination of PAHs (26 PAHs in total) and metals in mussel soft tissues was performed by Akvaplan-niva AS and the ALS laboratory group, respectively. Both laboratories are accredited for the methods used.

The analysis of total PAHs included quantification of single analytes such as naphthalene, phenanthrene/anthracene, dibenzothiophene, and their C1–C3 alkyl structural analogues (sum NPDs); and sixteen 3–6 ring aromatics listed as priority pollutants by the Environmental Protection Agency (16 EPA-PAHs). Two pools (5–8 mussels each) of mussel soft tissues from each site (only one pool from Buktalandet) were used for the quantitative determination of total PAHs. Each pool (11–23 g wwt) was weighed and a potassium hydroxide-methanol solution and an internal standard-mix of deuterated PAHs were added. The solution was boiled under reflux for 4 h (saponification), filtered and extracted with pentane. The extract was dried with Na_2SO_4 (s), concentrated to 0.5 ml and purified using gel permeation chromatography (GPC), with dichloromethane (DCM) as the mobile phase, to remove fats and other impurities. After evaporation to approximately 0.5 ml and solvent exchange to hexane, the extract was further purified on a solid phase extraction (SPE) column by elution with pentane and DCM. Eluted solution was concentrated to 0.5 ml, evaporated to dryness under N_2 gas, and finally 100 μl isooctane was added. Quantification of the final extract was carried out using isotope-labeled internal standard calibration. Analyses were performed using Gas Chromatography–Mass Spectrometry (GC–MS). Blind samples were run parallel to all samples, and proficiency test samples (Quasimeme, Netherlands) were used as controls. The limit of detection (LOD) was determined from analyses of a series of blank samples, processed along with real samples, and calculated as: LOD = (blank average) + 3 × (blank standard deviation). Half of the detection limit value in compounds that were below LOD was used to calculate sums of NPDs and 16 EPA-PAHs.

Metal concentrations were analysed in the third pool (5–8 mussels) of mussel soft tissues from each site. Frozen soft tissues (11–21 g wwt) were homogenized and then freeze-dried. The dried samples were dissolved in concentrated nitric acid and hydrogen peroxide by microwave digestion (170 °C, 30 min) in sealed Teflon vessels. Cooled samples were transferred to test tubes and diluted to 10 ml. The concentration of As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb and Zn were measured by inductively coupled plasma sector field mass spectrometry (ICP-MS). Analytical quality was confirmed through analyses of certified reference material bovine muscle powder (NIST 8414).

2.7. Biochemical analyses of mussel digestive gland tissues

Digestive gland (100 mg) was homogenized in 1.6 ml potassium phosphate buffer (100 mM, pH 7.5) with 2.5% NaCl using a precellys at 3 × 5000 rpm × 5 s. For all biomarkers except lipid peroxidation, the homogenates were subsequently centrifuged at 10000*g* for 30 min at 4 °C. The obtained supernatants were split into subsamples for different biomarkers and stored at −80 °C.

Lipid peroxidation, an indicator of oxidative stress on lipids in cells, was measured as thiobarbituric reactive substances (TBARS) (Buege and Aust, 1978). The standard curve of 1,1,3,3-tetramethoxypropane (0–5 μM) and sample homogenates were mixed with TCA-TBA reagent (trichloroacetic acid at 15% w/v, thiobarbituric acid at 0.375% w/v and HCl 37% diluted at 0.25 N) in glass reagent tubes and incubated at 100 °C for 15 min. Tube content was transferred to Eppendorf tubes and centrifuged at 1000*g* for 10 min (4 °C). The supernatant was pipetted in triplicates into a microplate. The absorbance was read at 532 and 600 nm with the spectrophotometer plate reader. The absorbance at 532 nm was normalised to the absorbance at 600 nm. The concentration was then calculated from the slope of the standard curve and expressed as nmol TBARS/mg cytosolic protein.

CAT is an antioxidant enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen and protects cells from the oxidative stress. CAT activity was evaluated by the method of Clairborne (1985), which is based on the disappearance of hydrogen peroxide (H_2O_2) with time by the action of CAT contained in the examined samples. The decrease in absorbance at 240 nm was recorded in a quartz cuvette every second for 1 min at 20 °C after addition of 10 mM H_2O_2 to the twice-diluted sample homogenate and 50 mM phosphate buffer (pH 7) using a spectrophotometer LAMBDA 35. CAT activity was measured in duplicate and expressed in μmol/min/mg cytosolic protein.

GST is a pollution biomarker in bivalves (Brooks et al., 2011). This enzyme is generally induced by oil-related compounds as it takes part in their detoxification. GST activity was determined by measuring the increase in absorbance at 340 nm with time due to the conjugation of glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) and the formation of S-2,4-dinitrophenyl under the catalyzation of GST enzyme (Habig et al., 1974). The absorbance was recorded in a quartz cuvette filled with twice-diluted sample homogenate, 20 mM CDNB, 20 mM GSH and 100 mM potassium phosphate buffer (pH 7.5) with 2.5% NaCl. Recording was made every second for 1 min at 20 °C. GST activity was measured in duplicate and expressed as nmol/min/mg cytosolic protein.

The cytosolic protein concentration of the homogenates (mg/ml) was determined according to Bradford (1976) using a bovine serum albumin standard curve (0–0.01 mg/ml). Standard solution or sample homogenates diluted 1000× were pipetted in triplicates into microwells. Coomassie blue reagent diluted 5× was added, and the plate was left for saturation for 2 min. Absorbance was measured at 595 nm using the PerkinElmer Victor 3 plate reader.

The TOSC assay provides a measure of the biological resistance to oxidative stress (Regoli and Winston, 1998). The TOSC was measured in digestive glands of the mussels from lower littoral zone by quantifying

the overall capability of cellular antioxidants to absorb artificially generated oxyradicals, such as peroxyl (ROO·) and hydroxyl (OH). The supernatants were ultracentrifuged at 50000 *g* for 120 min at 4 °C and the cytosolic fraction was stored at −80 °C until analysed. A separate reference was used for each sample, which was measured with each run on the gas chromatograph. TOSC was measured in 55 μg of cytosolic protein from each sample diluted in 800 μl of homogenizing buffer containing 2 mM α-keto-γ-methiolbutyric acid and 200 mM 2,2′azobis (2-methylpropionamidine) dihydrochloride. Glutathione was used as the positive control and homogenizing buffer was used as the blank. Ethylene gas production was measured at intervals of 12 min from 36 to 84 min. The gas was quantified by measurement with a GC-FID (6890N, Agilent Technologies, California, USA) and a Supelco SPB-1 fused silica capillary column (30 m × 0.32 mm × 0.25 μm). Hydrogen, at a flow rate of 1.5 ml/min, was the carrier gas with a split ratio of 1:20 with an inlet temperature of 160 °C, oven temperature of 72 °C and a FID temperature of 220 °C. The TOSC values were calculated using the equation: $TOSC = (100 - (\int SA/\int Blk \times 100)) / 55$, where "∫SA" is the integral of the curve for the sample from the GC readout, "∫Blk" is the integral of the curve for the blank from the GC readout. The TOSC values were normalised to cytosolic protein concentration and expressed as Unit TOSC/mg cytosolic protein. The cytosolic protein concentration of the samples was determined using the Lowry method (Lowry et al., 1951), adapted for measurement with a plate reader and using bovine gamma globulin as the protein standard.

2.8. Statistical analyses

Due to presence of multiple factors that potentially affected the seasonal variability of biomarkers, the multivariate analyses Principal component analysis (PCA) and Projections to Latent Structures (PLS) were performed on the data from the seasonal study using Simca P14.1 Software.

PCA was used for visualising the differences and similarities in the data set by calculating principal components. These are mutually orthogonal vectors that represent independent and uncorrelated variance of the initial descriptors (variables). Correlated descriptors are described by the same principal component. The systematic variance in the data set can hence be simplified by using fewer new descriptors than the original number of variables, and this simplification is done without loss of systematic information (Carlson and Carlson, 2005). Since values of the descriptors varied in magnitude, the data were logarithmically transformed and subsequently centred and scaled to unit variance in the calculated PCA models. The number of significant components was determined by cross-validation. R^2X is introduced as a measure of the fraction of the variation of X variables explained by the model, and Q^2 is an estimate of the reliability/stability of the model calculated by cross-validation. Score plots are obtained by projecting the original data onto the calculated orthogonal principal component vectors. Variables with similar variance in their characteristics are plotted close to one another. The influence of each original descriptor to the principal component is reflected in a loadings plot. Descriptors which have a strong contribution to the variation depicted in the score plot are found far from the origin in the loadings plot. Positively correlated descriptors are projected close to each other, while negatively are projected opposite to each other with respect to the axis centre.

The PLS models were run for all months, both tidal zones and both genders. Reproductive stage 0 and I were combined in early maturity phase, while stage II and III were combined in late maturity phase in order to increase number of mussels (n) per reproductive stage. Tidal zone, and individual parameters of the mussels (shell volume, CI, gender, maturity phase) were applied as predictor variables. Biomarkers (TBARS, CAT, GST, TOSC) were applied as responses.

The validity of the models was expressed by the amount of the variance in the response matrices ($R^2_{responses}$), which accordingly should be approaching 1. Calculated models with $R^2_{responses}$ values below 0.4 were disregarded. Q^2 is the predictive power, an estimate of the reliability/stability of the model calculated by cross-validation. In order to obtain a high predictive power, $R^2_{responses}$ should be high. A value of $Q^2 > 0.9$ is excellent, while a value above 0.5 is good. With non-significant components, Q^2 can even be negative. The difference between $R^2_{responses}$ and Q^2 should be as low as possible. The models with a difference in $R^2_{responses}$ and Q^2 larger than 0.3 were considered unstable, as it may indicate outliers or the presence of irrelevant predictor variables.

The output of the model included the variable importance in the projection (VIP) plots presenting the importance of each variable in the model with respect to its correlation to all the responses and to the projection. VIP plots reflected the relative importance of the model variables to each other. Variables with VIP values >1 were considered as variables with high influence on the model, they were relevant for explaining the responses; variables with VIP values in the range 0.5–1 were considered as variables with moderate influence on the model; variables with VIP values <0.5 were considered as variables with low influence on the model.

Microsoft Excel and software package XLSTAT Version 2016.03.30887 were used for statistical analyses among group means. Assumptions of normality and homogeneity of all variances were checked using normal P-plot and Levene's test, respectively. As neither assumption was violated, one-way ANOVA was performed. Statistical significance was considered when $p \le 0.05$.

For the seasonal study, significant differences ($p \le 0.05$) in individual parameters of the mussels (length, width, height, shell volume, CI) were tested across months (11), and for upper and lower littoral zones. Significant differences ($p \le 0.05$) in biomarkers (TBARS, CAT, GST, TOSC) were tested across months (11), for upper and lower littoral zones, for females and males, and for early and late maturity phase. Total Chl A concentrations in water samples were tested for significance across months (7).

For the spatial study, significant differences ($p \le 0.05$) in individual parameters of the mussels (length, width, height, shell volume, CI) and biomarkers (TBARS, CAT, GST) were tested across sites (6), for females and males, and across reproductive stages (4). Bioaccumulated concentrations of 16 EPA-PAHs and NPDs in mussel soft tissues, as well as total Chl A concentrations in water samples were tested for significance across sites (6). Statistical analyses were not run for metals as the samples included only one replicate per sampling site.

Tukey's post-hoc test was performed to make multiple pair wise comparisons and find which pairs of months, sites and reproductive stages were significantly different ($p \le 0.05$).

A chi-square test was used to compare observed sex ratios (number of females: number of males) in the mussels from each of the tidal zones and from each of the site of the spatial study to the theoretical proportion 1:1 (significance level $p \le 0.05$).

Possible differences ($p \le 0.05$) in observed distributions of reproductive stages (number of mussels per reproductive stage) and LMS (number of mussels per neutral red retention time class) were tested using a Fisher's exact test. For the seasonal study, the differences were tested across months, for upper and lower littoral zones, for females and males from the same tidal zone and across reproductive stages (only LMS). For the spatial study, the differences were tested across sites and for females and males at each site.

3. Results

3.1. Seasonal study

3.1.1. Environmental conditions in Rakkfjorden

With its location at the 69th parallel north of the Arctic Circle, both photoperiod and light intensity in Rakkfjorden vary significantly over the year. The highest light intensities (>10,000 lx) were recorded from the end of May to the end of July, when the Sun is continuously above the horizon (Fig. S3A). The lowest light intensities (<500 lx) were

recorded from the beginning of December to the beginning of February when the Sun is continuously below the horizon.

The warmest period in Rakkfjorden in 2013 was recorded from the middle of May to the middle of September, with weekly mean air temperatures ranging from 10 °C to 15 °C (Fig. S3A). The lowest weekly mean air temperatures were recorded in December (−4 °C), January (−11 °C) and March (both years of logging −6 °C).

The weekly mean water temperature increased from 2.0 ± 0.1 °C in March to 11.7 ± 0.3 °C in June, July and August, and the maximum water temperature (13.5 ± 0.1 °C) was recorded at the end of July (Fig. S3B). There was a gradual decrease in water temperature from 10.8 ± 0.4 °C in September to 0.6 ± 0.4 °C at the end of January.

Salinity changed both over time and with the tidal cycle due to the location of the mussel bed between the fjord with seawater and a lagoon with freshwater, from snow melt and land runoff. In February and March, salinity was relatively stable at 30.2 ± 0.1 psu because the lagoon was frozen, but in July, August and September, salinity fluctuated between 24.1 ± 0.5 psu during rising tides and 15.8 ± 0.6 psu during falling tides (Fig. S4).

3.1.2. Total Chl A concentration in seawater

Significantly high levels of Chl A in seawater in Rakkfjorden were recorded in March and April (0.9–1.4 mg/m^3; Fig. S5). The levels of primary production (0.5–0.7 mg/m^3) remained elevated in May, June and September. In January, the total Chl A concentration was the lowest (0.069 ± 0.003 mg/m^3).

3.1.3. Description of collected mussels

Mussels in this study were selected by hand to be within the largest size range of their specific tidal zone. Thus, mussels from the lower littoral zone were significantly larger than mussels from the upper littoral zone in all months, except August, November and December (Table 1; Table S2). Furthermore, mussels from the lower littoral zone had significantly higher CI than mussels from the upper littoral zone in the winter and early spring month (April 2013, December, January, March and April 2014) (Table 1). However, no major significant differences in shell volume and CI were noted in any mussels across months (Table 1).

The sex ratios of the mussels from the upper littoral zone and from the lower littoral zone were balanced (Table S1) with no significant differences between months (data not shown). Gender related differences were only found sporadically for shell volume and CI in July (data not shown). The distributions of the reproductive stages in the population of collected mussels (females and males together) was similar ($p > 0.05$) between tidal zones for all months, except in July, where a higher proportion (70%) of stage 0 individuals were collected from the upper littoral zone, compared to the lower littoral zone (13%) (Fig. 2). The appearance of male mussels with mature gonads (stage III) was recorded in January and April 2014 for the lower littoral zone, whereas in the upper littoral zone it was firstly recorded in May.

The mussels had mature gonads (stage III) between April (lower littoral zone)/May (upper littoral zone) and July and spawned between June and September (Fig. 2). In June and September, gonads were spent (stage 0). Gonads in early gametogenesis (stage I) were observed in all months, except June, and gonads in active gametogenesis (stage II) were present in all months, except September.

3.1.4. Biomarkers

There was a significant seasonal variation in LMS in mussel haemocytes from both tidal zones, with higher neutral red retention time (90–180 min) in the summer half-year (April to September) than in the winter half-year (November, December and March, 0–60 min) (Fig. 3). No differences in LMS were found for mussels from the upper and lower littoral zones in any month, neither across reproductive stages in any month.

Table 1
Mean (±SE) volume and condition index (CI) of the mussels *Mytilus* spp. from upper and lower littoral zones (n = 9–12 per month from each tidal zone) collected for the seasonal study in Rakkfjorden. Asterisk (*) for the mussels from lower littoral zone indicate significant differences (ANOVA, $p \le 0.05$) with the mussels from upper littoral zone. Letters (a, b, c) indicate significant differences (ANOVA, $p \le 0.05$) across months. No significant differences (ANOVA, $p \le 0.05$) in CI were found for mussels from upper littoral zone across months.

Month	Upper littoral zone		Lower littoral zone	
	Volume, ml	CI, %	Volume, ml	CI, %
April 2013	4.9 ± 0.3^{a,b}	33.8 ± 1.7	6.0 ± 0.2^{*,b}	39.1 ± 1.8^{*,a,b}
May	4.1 ± 0.3^{b}	37.8 ± 2.4	7.2 ± 1.0^{*,a,b}	37.2 ± 2.9^{b}
June	4.7 ± 0.4^{a,b}	37.5 ± 2.4	7.4 ± 0.7^{*,a,b}	42.0 ± 1.8^{a,b}
July	3.8 ± 0.3^{b}	37.6 ± 1.3	6.7 ± 0.7^{*,a,b}	42.4 ± 3.5^{a,b}
August	4.9 ± 0.4^{a,b}	35.8 ± 1.5	3.0 ± 0.4^{*,c}	39.5 ± 1.8^{a,b}
September	4.4 ± 0.1^{a,b}	38.1 ± 1.2	7.0 ± 0.6^{*,a,b}	39.4 ± 1.3^{a,b}
November	4.8 ± 0.3^{a,b}	36.7 ± 1.2	5.7 ± 0.4^{b,c}	36.6 ± 1.2^{b}
December	5.9 ± 0.5^{a}	37.1 ± 2.7	5.0 ± 0.4^{b,c}	46.8 ± 1.1^{*,a}
January	4.8 ± 0.2^{a,b}	32.9 ± 1.2	9.6 ± 1.1^{*,a}	42.9 ± 2.0^{*,a,b}
March	4.1 ± 0.4^{b}	31.0 ± 1.0	7.3 ± 0.4^{*,a,b}	41.6 ± 1.1^{*,a,b}
April 2014	3.7 ± 0.4^{b}	36.1 ± 1.5	7.7 ± 0.8^{*,a,b}	43.0 ± 0.7^{*,a,b}

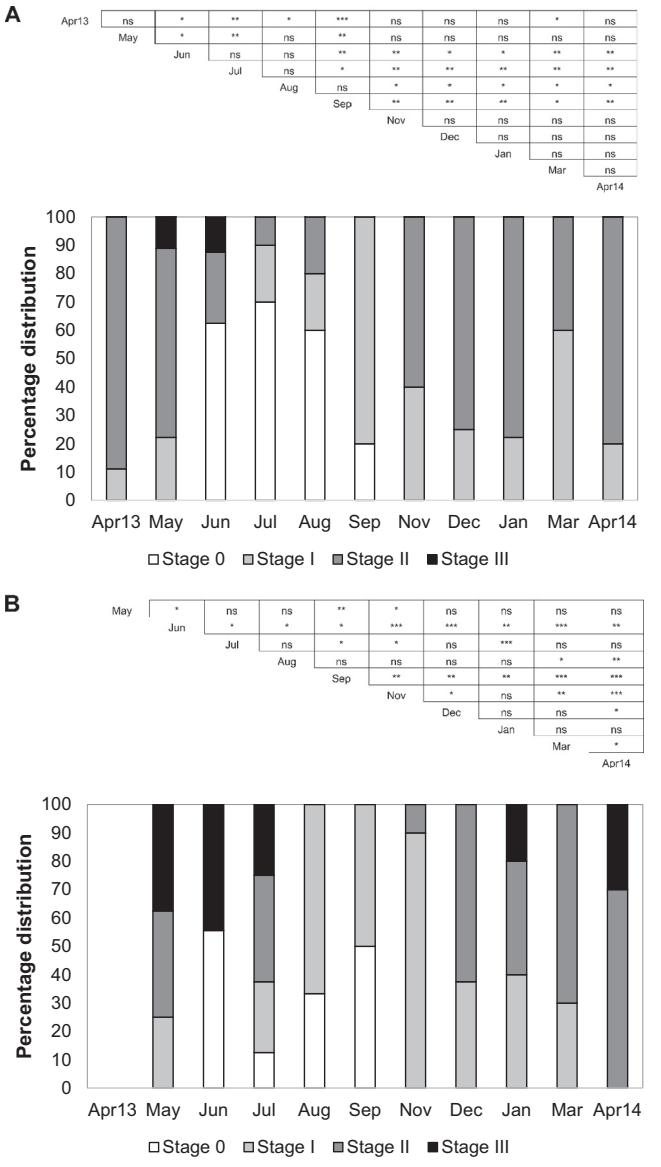
A
Apr13 May Jun Jul Aug Sep Nov Dec Jan Mar Apr14
Percentage distribution
Stage 0 Stage I Stage II Stage III
B
Apr13 May Jun Jul Aug Sep Nov Dec Jan Mar Apr14
Percentage distribution
Stage 0 Stage I Stage II Stage III

Fig. 2. Distribution of reproductive stages of mussels *Mytilus* spp. from upper (A) and lower (B) littoral zones (n = 9–12 per month from each tidal zone) collected for the seasonal study in Rakkfjorden. The inserted table indicates significant differences (Fisher's exact test; ns = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001) across months.

Only a few differences in biomarkers, such as TBARS, CAT, GST and TOSC, were found among months, between tidal zones, between genders, and between early and late maturity phase. As part of an initial assessment, PCA models were used to visualise potential differences and similarities between exogenous variables (month, light intensity, water temperature, salinity, Chl A, tidal zone), endogenous variables (shell volume, CI, gender, reproductive stage/maturity phase) and the biomarkers. Including all variables and responses resulted in poor PCA models, describing <40% of the variance in the dataset. For this reason, variables and responses were analysed separately. The first PCA model included all the exogenous and endogenous variables and the X-axis explained 53% of their variance (Fig. 4A) with clusters according to the months (Class 1–11). The loadings plot revealed that exogenous factors (light intensity, water temperature, salinity and Chl A) had a strong influence on the variance in X-axis (Fig. 4B). Since these exogenous factors were equal on the same month, these variables might result in skewed representation of the variance in the PCA model. Based on this, a new PCA model was calculated, which only included variables that varied on and between the months (CI, shell volume, tidal zone, reproductive stage/maturity phase and gender). The model explained 56% of the variance and no clustering according to the month was observed (Fig. 4C). The loadings plot showed that the tidal zone, CI and shell volume had the highest influence on the variance in X-axis (explaining 29% of the variance) and reproductive stage/maturity phase had the highest influence on the variance in Y-axis (explaining 27% of the variance) (Fig. 4D). The PCA model of the responses explained 54% of the variance and there was clustering according to the month (Fig. 4E). This indicated that the trend in responses varied according to the biomarker. TBARS and CAT had the highest influence on the variance in X-axis, while TOSC-ROO· had the highest influence on the variance in Y-axis (Fig. 4F).

The PLS models were plotted to visualise effect of the tidal zone/shell volume/CI, gender and maturity phase on each of the biomarkers.

In the model for TBARS, the gender was identified as an important factor for the biomarker in June and December (Fig. 5). In June, males from the upper littoral zone had significantly higher (1.2 ± 0.1 nmol/mg protein) TBARS levels than females (0.69 ± 0.04 nmol/mg protein). However, no significant differences in TBARS between genders were found in December. High importance of the maturity phase as variable in the model was identified in mussels in March (Fig. 5), when in the upper littoral zone males in the early maturity phase had lower TBARS levels (stage I; 0.6 ± 0.1 nmol/mg protein) than males in the late maturilty phase (stage II; 1.2 nmol/mg protein). Also, the PLS indicated that the tidal zone and the CI would affect the TBARS levels in December, even though the difference was weak and not significant ($p > 0.05$) based on the ANOVA (Fig. 6A). The seasonal trend for TBARS levels was similar for mussels from both tidal zones, with highest TBARS levels observed in May (3.4 ± 0.7 nmol/mg protein) (Fig. 6A). In all other months, the TBARS levels were not significantly different from each other (mean of 0.91 ± 0.04 nmol/mg protein).

In the model for CAT activity, maturity phase and tidal zone had a high influence on the biomarker in July and November (Fig. 5). Significant differences in CAT activity in relation to these two factors were found only in mussels collected in November. Indeed, in the upper littoral zone, males in early maturity phase had higher (26.2 μmol/min/mg protein) CAT activity than males in late maturity phase (5.3 ± 1.6 μmol/min/mg protein). Also, higher CAT activity was measured in the mussels from the lower littoral zone compared to the upper littoral zone only in November (Fig. 6B). CAT activity was relatively stable across months. The only remarkable change in CAT activity was significantly increased levels in mussels from the upper littoral zone in April 2013 (120.7 ± 43.7 μmol/min/mg protein) compared to August, September, November, March and April 2014 (mean of 10.4 ± 1.2 μmol/min/mg protein; Fig. 6B).

In the model for GST activity, high importance of the tidal zone/shell volume/CI was recorded in April 2013 and January (Fig. 5). Indeed, the GST activity was higher in mussels with smaller shell volume and lower CI (Table 1) from the upper littoral zone (upper 29.2 ± 2.8 nmol/min/mg protein vs. lower 18.3 ± 3.1 nmol/min/mg protein) (Fig. 6C). No significant differences in the GST activity were observed in any tidal zone across months (mean of 27.4 ± 1.2 nmol/min/mg protein).

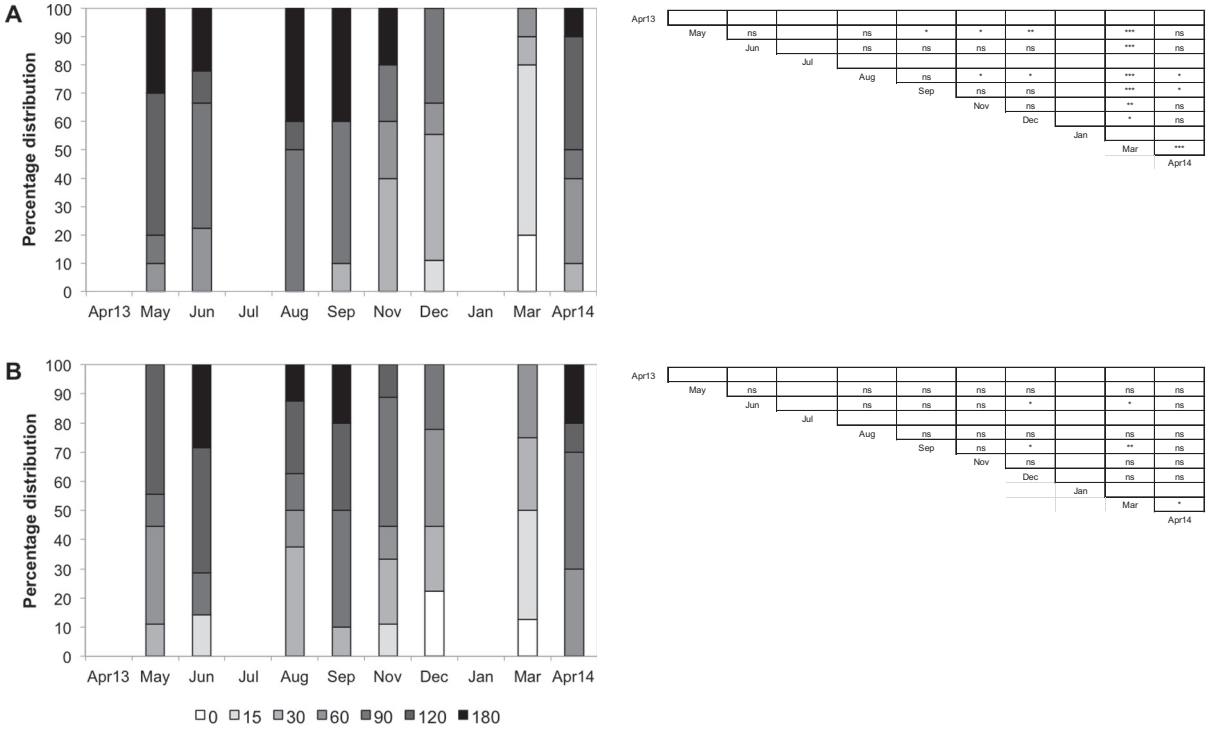

Fig. 3. Distribution of lysosome membrane stability (LMS) measured as neutral red retention time (min) in haemocytes of mussels *Mytilus* spp. from upper (A) and lower (B) littoral zones (n = 9–12 per month from each tidal zone) collected for the seasonal study in Rakkfjorden. The inserted tables indicate significant differences (Fisher's exact test; ns = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001) across months.

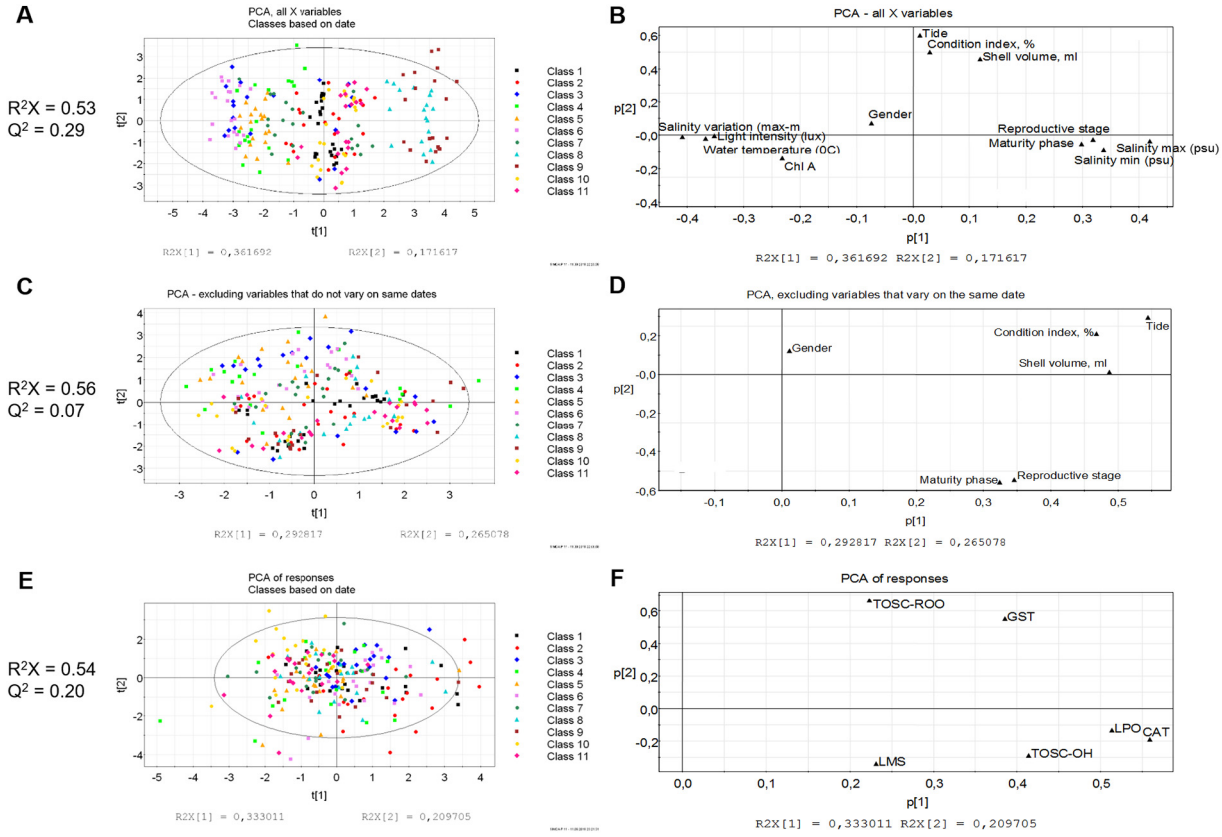

Fig. 4. Results of the principal component analysis (PCA) models: scores plots (A, C, E) and loadings plots (B, D, F). R^2X indicates a measure of the fraction of the variation of X variables explained by the model. Q^2 is an estimate of the reliability/stability of the model calculated by cross-validation.

In the models for TOSC, the importance of the variables was slightly different between two radicals depending on the month (Fig. 5). However, it seemed that all individual parameters of the mussels (CI, shell volume, gender and maturity phase) had high influence on the biomarker. TOSC values for neutralisation of the ROO· radical (TOSC-ROO·) were not significantly different across months (mean of 460.9 ± 6.2 U/mg protein; Fig. 6D). TOSC values for neutralisation of the OH radical (TOSC-OH) was higher in mussels collected in May (489.0 ± 37.4 U/mg protein) than in April 2013, June, July, November, January, March and April 2014 (mean of 310.5 ± 9.4 U/mg protein) (Fig. 6E).

3.2. Spatial study

3.2.1. Environmental conditions

Similar weather conditions (wind of 2–5 m/s, low-level clouds, fog and drizzle) and air temperatures (12–16 °C) were recorded at all sites for all sampling days. Water temperature was lower in Simaviknes (9.2 °C) and Oldervik (10.4 °C) than at the other sites (mean of 14.6 ± 0.9 °C), possibly because the sampling was done during the morning hours (Table S3). Salinity of 31–34 psu was measured in Simaviknes, Rakkfjorden, Tromvik and Brensholmen (Table S3). Salinity was low in Oldervik (5 psu) and Buktalandet (7 psu) due to the arrival of freshwater from the land runoff and river, respectively (Table S3).

3.2.2. Total Chl A concentration in seawater

The highest concentration of Chl A (8.2 ± 0.1 mg/m^3) was measured in Simaviknes (Fig. S6). At this site, a high concentration of green algae were observed in the water. In Tromvik, Brensholmen and Buktalandet, the Chl A ranged between 2.2 and 2.9 mg/m^3. In Oldervik and Rakkfjorden, the Chl A concentrations were the lowest (1.1–1.5 mg/m^3).

3.2.3. Description of collected mussels

The sex ratio was even among the mussels collected in Simaviknes (females n = 7, males n = 8) and Oldervik (females n = 8, males n = 7). Females were dominant in samples collected in Tromvik (females n = 11, males n = 4), whereas there were more males than females in Rakkfjorden (females n = 4, males n = 11), Brensholmen (females n = 5, males n = 10) and Buktalandet (females n = 5, males n = 10). No differences in shell volume and CI were found between genders at any site.

Mussels collected in Oldervik, Tromvik and Brensholmen were significantly larger in volume (min–max 11.0–28.3 ml) than mussels collected from the other three sites (min–max 7.9–18.3 ml; Table 2). The CI tended not to be different across the sites (Table 2).

The significant dominance of individuals at stage 0 was observed for females (n = 3) and males (n = 7) from Rakkfjorden, females from Simaviknes (n = 4) and females from Buktalandet (n = 5) (Fig. 7). Stage I was presented in most of the mussels from Brensholmen (females n = 4, males n = 9), Oldervik (females n = 6, males n = 6) and in males from Buktalandet (n = 9). Individuals at stage II were dominated among males from Simaviknes (n = 5). Distributions of the reproductive stages were similar for both genders from Tromvik.

Distributions of reproductive stages of females and males were significantly different only in Buktalandet (females (n = 5) were at stage 0, whereas males were at stage 0 (n = 1) and I (n = 9)) (Fig. 7).

No differences in shell volume or CI were found across reproductive stages at any site.

3.2.4. Contaminant burden in total soft tissues of the mussels

No significant differences in concentration of 16 EPA-PAHs in mussel soft tissues were found across sites (Table 3). According to the Norwegian Environmental Agency classification of environmental quality

TBARS

	Apr13	May	Jun	Jul	Aug	Sep	Nov	Dec	Jan	Mar	Apr14
$R^2_{responses}$			0.6					0.4		0.4	
Q^2			0.2					−0.1		−0.1	
Condition index			0.4					1.4		0.3	
Gender			1.9					1.3		0.7	
Maturity phase			0.6					0.8		1.0	
Shell volume			0.5					0.6		0.0	
Tidal zone			0.6					1.3		0.4	

CAT

	Apr13	May	Jun	Jul	Aug	Sep	Nov	Dec	Jan	Mar	Apr14
$R^2_{responses}$				0.5			0.6				
Q^2				0.5			0.4				
Condition index				0.1			0.6				
Gender				0.9			0.7				
Maturity phase				1.0			1.6				
Shell volume, ml				0.7			0.0				
Tidal zone				1.1			1.1				

GST

	Apr13	May	Jun	Jul	Aug	Sep	Nov	Dec	Jan	Mar	Apr14
$R^2_{responses}$	0.4								0.5		
Q^2	0.2								0.2		
Condition index	0.7								1.4		
Gender	0.1								0.9		
Maturity phase	0.7								0.1		
Shell volume	1.7								1.2		
Tidal zone	1.3								1.5		

TOSC-ROO

	Apr13	May	Jun	Jul	Aug	Sep	Nov	Dec	Jan	Mar	Apr14
$R^2_{responses}$	0.7		0.7			0.7	0.5		0.7	0.4	
Q^2	0.7		0.1			0.0	0.1		0.4	0.0	
Condition index	1.6		0.8			1.2	1.5		1.7	0.7	
Gender			2.1			0.4	0.6		1.2	1.7	
Maturity phase			0.6				1.4		1.3	0.4	
Shell volume	1.8		0.9			1.4	0.2		0.3	1.5	

TOSC-OH

	Apr13	May	Jun	Jul	Aug	Sep	Nov	Dec	Jan	Mar	Apr14
$R^2_{responses}$		0.7	0.3	0.8				0.4			
Q^2		0.5	0.1	−0.1				−0.1			
Condition index		1.3	1.1	1.0				1.5			
Gender		1.2	0.1	1.3				0.9			
Maturity phase		1.2	0.6	1.3				1.1			
Shell volume		0.7	1.9	0.7				1.1			

Fig. 5. Results of the projections to latent structures (PLS) models presented as variable importance in the projection (VIP) values. The models were run for all data of the seasonal study. Tidal zone and individual parameters of the mussels (condition index, gender, maturity phase and shell volume) were applied as predictor variables. Biomarkers (lipid peroxidation measured as thiobarbituric reactive substances (TBARS), catalase (CAT) activity, glutathione S-transferase (GST) activity, total oxyradical scavenging capacity toward ROO· radical (TOSC-ROO·) and OH radical (TOSC-OH)) were applied as responses. Black cells indicate variables with high influence on the model with VIP values >1; grey cells indicate variables with moderate influence on the model with VIP values in the range 0.5–1; light grey cells indicate variables with low influence on the model with VIP values <0.5; white cells indicate poor models or no data. $R^2_{responses}$ indicates amount of variance explained in the response matrices, i.e. biomarkers (TBARS, CAT, GST, TOSC-ROO·, TOSC-OH). Q^2 is an estimate of the reliability/stability of prediction of the PLS model calculated by cross-validation.

status based on the contaminant burden in blue mussels (Molvær et al., 1997), the degree of pollution of the sum 16 EPA-PAHs in all sites was ranked as moderate (Class II – no toxic effects). The levels of NPDs bioaccumulated in mussels were similar at all sites, with the highest concentration in Brensholmen (Table 3). There is no quality standard including background levels of NPDs.

The levels of metals in mussel soft tissues were relatively similar across all sites too. Zn was the dominant metal followed by As, Cu and Mn (Table 3). The degrees of pollution of Cr, Hg, Ni and Pb in Rakkfjorden, Simaviknes and Buktalandet were ranked as moderate (Class II – no toxic effects), while As, Cd, Cu and Zn were ranked as marked (Class III – chronic effects) (Molvær et al., 1997). The site in

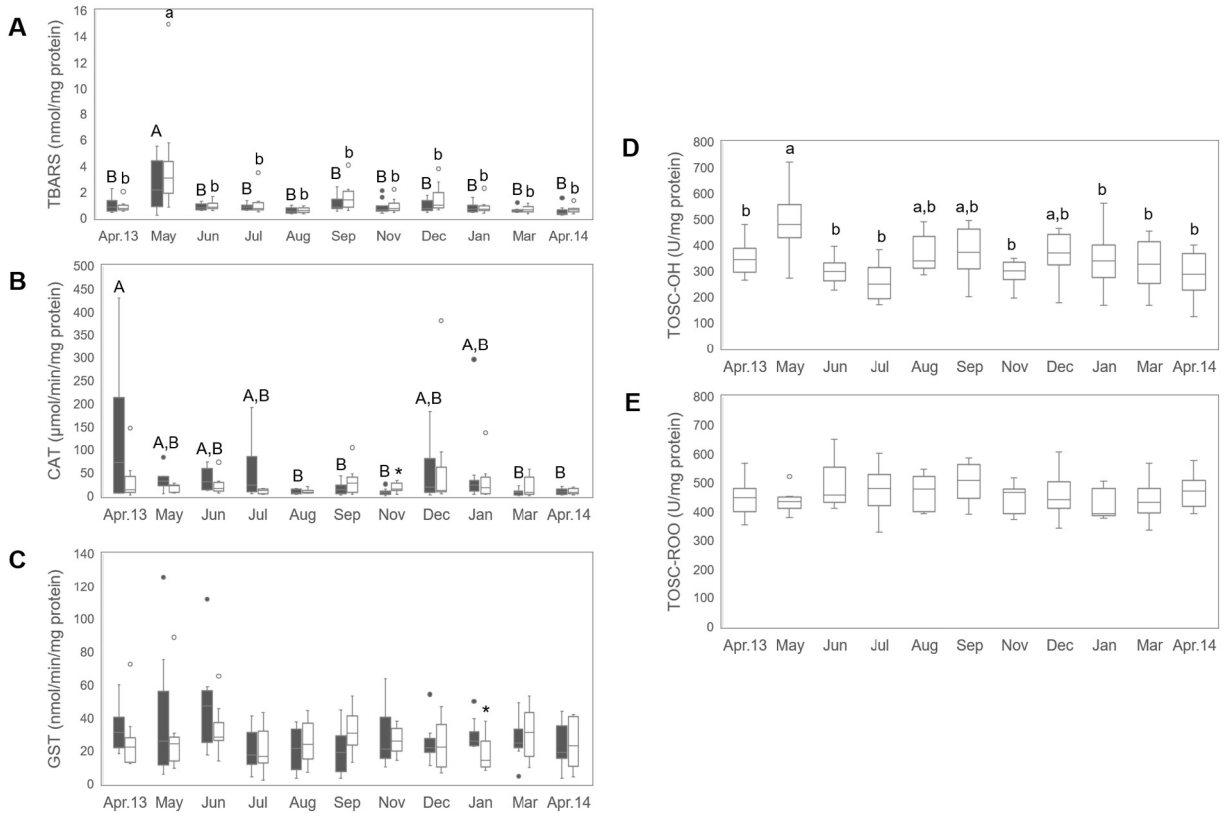

Fig. 6. Biomarkers measured in digestive gland of the mussels *Mytilus* spp. from upper littoral zone (grey) and lower littoral zone (white) (n = 9–12 per month from each tidal zone) collected for the seasonal study in Rakkfjorden. (A) Lipid peroxidation measured as thiobarbituric reactive substances (TBARS); (B) catalase (CAT) activity; (C) glutathione S-transferase (GST) activity; total oxyradical scavenging capacity (TOSC) toward (D) peroxyl radical (TOSC-ROO·) and (E) hydroxyl radical (TOSC-OH). Plots represent the median (line), 25%–75% percentiles (box), min-max range (whisker) and moderate outliers (dots). Asterisk (*) for mussels from the lower littoral zone indicate significant differences (ANOVA, $p \leq 0.05$) with mussels from the upper littoral zone. No significant differences (ANOVA, $p \leq 0.05$) in TBARS were found between mussels from the upper littoral zone and mussels from the lower littoral zone. Letters (A, B for mussels from the upper littoral zone, and a, b for mussels from the lower littoral zone) indicate significant differences (ANOVA, $p \leq 0.05$) across months. No significant differences (ANOVA, $p \leq 0.05$) in GST were found in mussels from the upper littoral zone across months. No significant differences (ANOVA, $p \leq 0.05$) in CAT, GST and TOSC-ROO· were found in mussels from the lower littoral zone across months.

Tromvik differed from Rakkfjorden only in reduced concentration of Zn (Class II – no toxic effects). The mussels from Buktalandet had elevated levels of Pb (Class III – chronic effects) and reduced levels of Zn (Class II – no toxic effects) compared to the mussels from Rakkfjorden. The site in Brensholmen was the most different in metal levels compared to Rakkfjorden, showing lower levels of Cr (insignificant degree of pollution, Class I – background level), Cd, Cu and Zn (Class II – no toxic effects), but higher levels of Pb (Class III – chronic effects). There are no quality standards including background levels of Co and Mn.

3.2.5. Biomarkers

TBARS levels, CAT activity and GST activity measured in mussels from Rakkfjorden in the spatial study were in the same range as in mussels collected for the seasonal study from the lower littoral zone in Rakkfjorden in July and August the year before.

Table 2
Mean (±SE) volume and condition index (CI) of mussels *Mytilus* spp. (n = 15 from each site) collected in six sites of the spatial study. Letters (a, b, c) indicate significant differences (ANOVA, $p \leq 0.05$) across sites.

Site	Volume, ml	CI, %
Simaviknes	12.8 ± 0.7^{b}	47.5 ± 1.1^{a}
Rakkfjorden	11.0 ± 0.7^{b}	$45.6 \pm 2.1^{a,b}$
Tromvik	18.8 ± 1.2^{a}	$42.4 \pm 0.8^{a,b,c}$
Brensholmen	16.7 ± 0.9^{a}	$41.9 \pm 1.2^{b,c}$
Oldervik	17.7 ± 0.8^{a}	38.7 ± 1.2^{c}
Buktalandet	11.4 ± 0.5^{b}	$44.9 \pm 1.3^{a,b}$

No significant differences in TBARS levels were observed across sites (Fig. 8A). TBARS levels did not differ between genders or across reproductive stages at any site.

CAT activity was not different across sites (Fig. 8B), between genders at any site, or across reproductive stages at any site.

GST activity was highest in mussels from Rakkfjorden (31.3 ± 12.7 nmol/min/mg protein) (Fig. 8C), however these results were only based on two male individuals, as all the other mussels from this site showed GST activity below the assay's limit of detection. No significant differences in GST activity were found between genders or across reproductive stages at any site.

4. Discussion

4.1. Seasonal study

The present study provides a relatively large dataset on the baseline levels of commonly used biomarkers (TBARS, CAT, GST, TOSC and LMS) in Arctic *Mytilus* spp. and their variations across seasons. In addition, it gives a description of the annual reproductive cycle of *Mytilus* spp. and their morphological and physiological features related to the location within the tidal zone. The obtained knowledge is important for determination of the natural range of biomarker baseline variability at different seasons and tidal conditions. Moreover, when assessing the acute exposure and following effects of a pollution event, the biomarker results should be adjusted to the pollution history of the considered site and biomarker baseline levels in the same month/season as the incident occurred. This will help to draw a picture as correct as possible of the

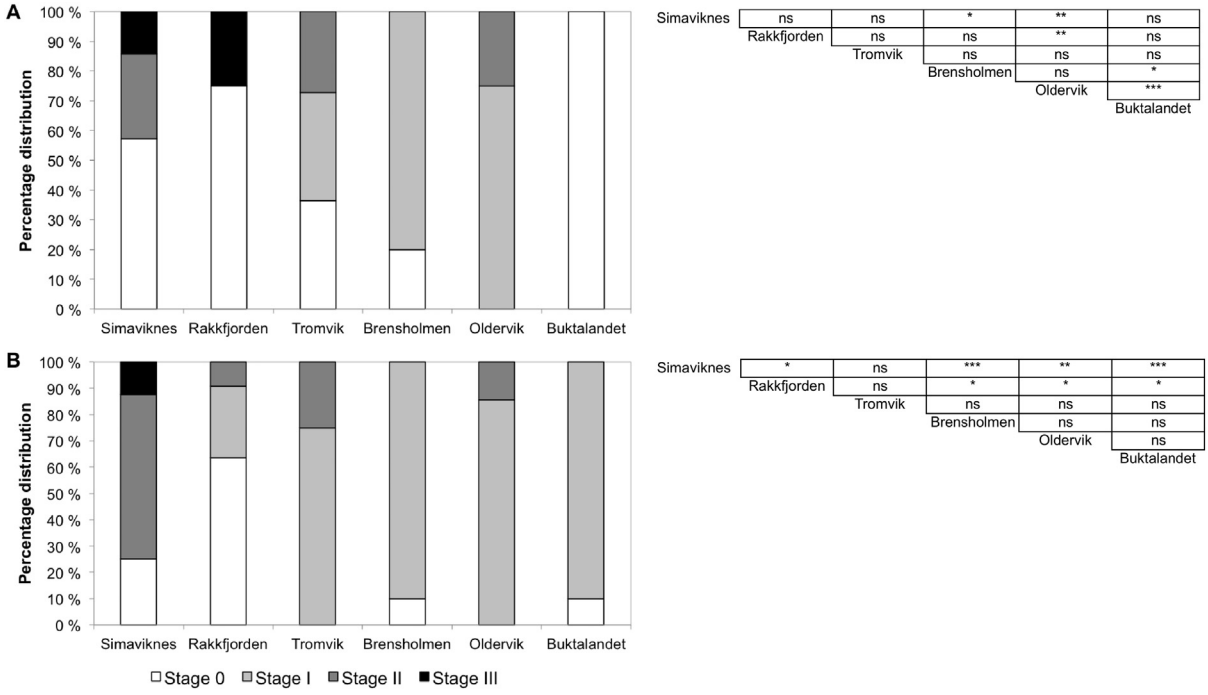

Fig. 7. Distribution of reproductive stages of female (A) and male (B) mussels *Mytilus* spp. (n = 15 per site) collected in six sites of the spatial study. The inserted tables indicate significant differences (Fisher's exact test; ns = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001) across sites.

environmental situation and help decision making during the recovery process.

Spawning period in Rakkfjorden took place between June and September, as individuals at stage 0 appeared among collected mussels. It is widely known that the reproduction of the mussels is closely associated with the water temperature (Chipperfield, 1953; Okaniwa et al., 2010). The temperature range between 9.5 and 12.5 °C triggers mussels to start spawning (Chipperfield, 1953). The water temperature in Rakkfjorden was 10 to 14 °C, when mussels spawned. As for the relationship between the highest concentration of phytoplankton in water and start of spawning, it is reported to vary (Thorarinsdóttir et al., 2013). In Rakkfjorden, the Chl A-bloom was observed in March, some months before spawning. The mussels were provided with food from the beginning of March to the end of September. In Iceland, some *M. edulis* spawned during the phytoplankton bloom, however, some of the mussels could start reproduction two weeks before the bloom (Thorarinsdóttir et al., 2013).

Spawning in mussels from Rakkfjorden was partial, no fully spent individuals were present in the samples in June and July and some mussels were at stages I and II in July and August. These mussels seemed to save some energy to mature for a secondary spawning in August and September, similar to the mussels from Western Norway (Duinker et al., 2008). However, the environmental conditions and food availability were likely not favorable for the secondary spawning event in Rakkfjorden as a few males with mature gonads (stage III) were observed in January. Their gametes would have to be resorbed, as this phenomenon is quite frequent in mussels (Pipe, 1987; Suárez et al., 2005; Thorarinsdóttir et al., 2013).

Gametogenesis in mussels from Rakkfjorden started when water temperature became lower (Thorarinsdóttir et al., 2013) and occurred throughout winter from November to March. In this period no individuals at stage 0 were observed. Fully matured gonads were developed by early spring, similarly to the known reproductive development of the *Mytilus* spp. from more temperate regions and, first described by Seed (1969) in British waters.

Table 3
Sum of 16 priority polycyclic aromatic hydrocarbons listed by the Environmental Protection Agency (16 EPA-PAHs), sum of naphthalene, phenanthrene/anthracene, dibenzothiophene, and their C1–C3 alkyl structural analogues (NPDs) (both n = 2 pools of 5–8 mussels from each site, except n = 1 from Buktalandet, mean ± SE, μg/kg soft tissue wet weight) and metals (n = 1 pool of 5–8 mussels from each site, mean ± SD, mg/kg soft tissue wet weight) in soft tissue pools from mussels *Mytilus* spp. collected in six sites of the spatial study. Letters (a, b) indicate significant differences (ANOVA, p ≤ 0.05) across sites. No significant differences (ANOVA, p ≤ 0.05) were found for 16 EPA-PAHs across sites. Statistical analyses were not run for metals.

	Simaviknes	Rakkfjorden	Tromvik	Brensholmen	Oldervik	Buktalandet
Sum 16 EPA-PAHs	102 ± 26	82 ± 16	54 ± 2	99 ± 5	97 ± 38	79
Sum NPDs	235 ± 21[b]	389 ± 71[b]	231 ± 37[b]	849 ± 93[a]	470 ± 78[a,b]	601[a,b]
As	13.4 ± 3.6	19.1 ± 5.1	16.8 ± 4.5	14.2 ± 3.8	9.4 ± 2.5	11.2 ± 3.0
Cd	1.5 ± 0.3	1.2 ± 0.2	1.8 ± 0.3	0.9 ± 0.2	1.2 ± 0.2	1.3 ± 0.2
Co	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.9 ± 0.2
Cr	0.7 ± 0.2	1.1 ± 0.3	0.9 ± 0.2	0.5 ± 0.1	1.0 ± 0.3	1.2 ± 0.3
Cu	6.4 ± 1.2	6.0 ± 1.1	7.5 ± 1.4	5.8 ± 1.1	6.5 ± 1.2	7.7 ± 1.4
Hg	0.05 ± 0.02	0.14 ± 0.05	0.09 ± 0.03	0.05 ± 0.02	0.04 ± 0.02	0.07 ± 0.02
Mn	4.4 ± 0.8	4.6 ± 0.9	4.1 ± 0.8	3.6 ± 0.7	6.9 ± 1.3	9.8 ± 1.8
Ni	1.0 ± 0.3	1.6 ± 0.4	2.4 ± 0.6	1.4 ± 0.4	1.0 ± 0.3	2.3 ± 0.6
Pb	1.4 ± 0.3	0.9 ± 0.2	1.0 ± 0.2	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
Zn	108 ± 21	100 ± 20	69 ± 14	64 ± 13	101 ± 20	74 ± 14

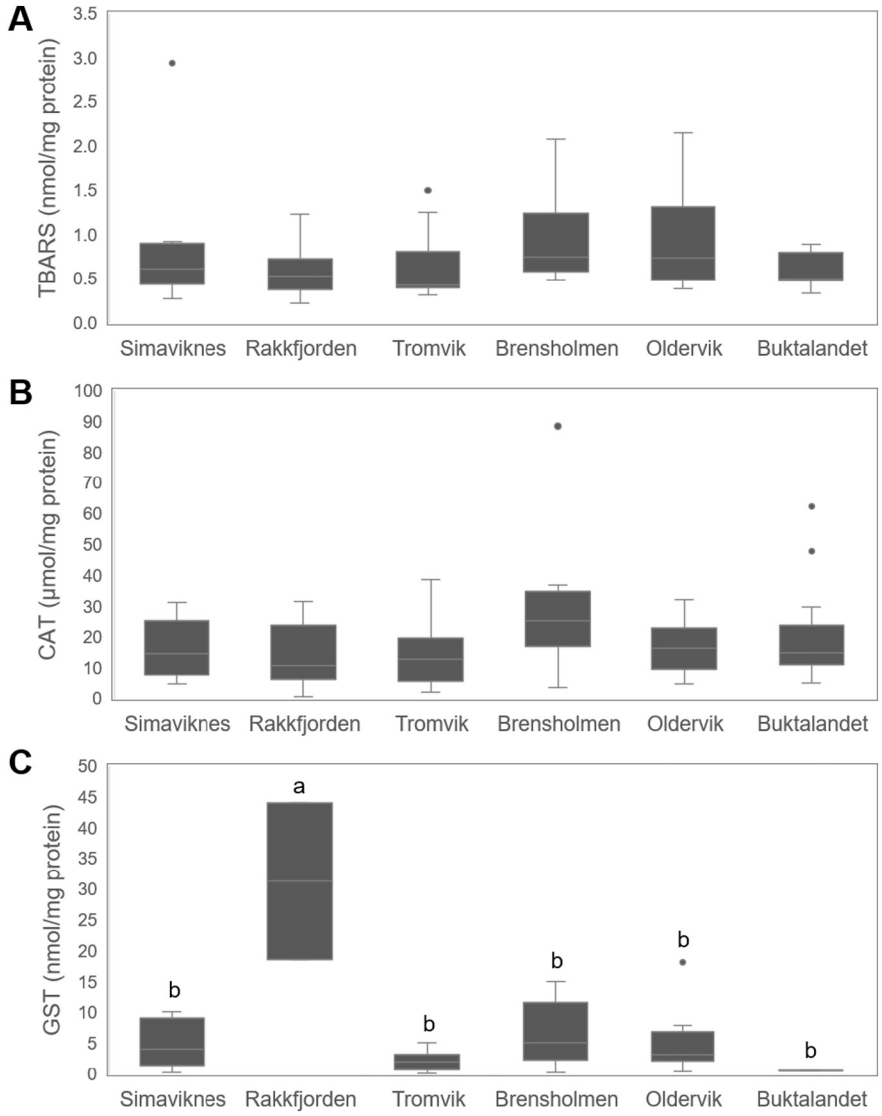

Fig. 8. Biomarkers measured in digestive glands of the mussels (n = 15 per site) collected in six sites of the spatial study: (A) Lipid peroxidation measured as thiobarbituric reactive substances (TBARS); (B) catalase (CAT) activity; (C) glutathione S-transferase (GST) activity. Plots represent the median (line), 25%–75% percentiles (box), min-max range (whisker) and outliers (dots). Letters (a, b) indicate significant differences (ANOVA, $p \leq 0.05$) across sites, with TBARS and CAT activity being not significantly different across sites.

The sampling site in Rakkfjorden was considered to be a good reference site as the bioaccumulated levels of contaminants (PAHs and metals) in mussels there were reported to be low (Nahrgang et al., 2013), with the site characterized as insignificantly polluted (Class I – background levels; Molvær et al., 1997).

The baseline levels of TBARS in mussels from Rakkfjorden were in the same range as in *Mytilus* spp. from a pristine location in Ireland (Schmidt et al., 2012; Schmidt et al., 2013) and the control *M. galloprovincialis* from an unpolluted site in North West Spain (Gonzalez-Fernandez et al., 2016). The range of CAT in the present study was similar to CAT measured in *M. edulis* from the northern Baltic Sea (Leiniö and Lehtonen, 2005) and the control *M. galloprovincialis* from an unpolluted site in North West Spain (Gonzalez-Fernandez et al., 2016). GST levels were also in the same range as in Gonzalez-Fernandez et al. (2016), however, they were lower than reported in Leiniö and Lehtonen (2005); Nahrgang et al. (2013) and Schmidt et al. (2013). The range of TOSC levels and the seasonal trend from this study were similar to the study by Nahrgang et al. (2013).

According to the results of the PCA models, reproductive stage/maturity phase of the mussels was found to be one of the most important drivers of the baseline levels of the studied biomarkers. Spawning during spring time in mussels from Rakkfjorden was associated with high levels of oxidative stress biomarkers and LMS. Indeed, CAT activity was high prior to spawning in April 2013; highest levels of TBARS and TOCS-OH were measured in ripe mussels that started to spawn in May; the levels of LMS stayed high during the whole spawning period. High levels of TBARS, CAT and TOSC associated with spawning during spring time were also reported in Cancio et al. (1999); Bocchetti and Regoli (2006); Schmidt et al. (2013) and Gonzalez-Fernandez et al. (2016), however, Hagger et al. (2010) showed a sharp decrease in LMS coincided with mussel spawning activity.

During the period of gonadal restoration after spawning, *Mytilus spp.* from Rakkfjorden tended to have decreased activities of antioxidant enzymes (TBARS, CAT and TOSC) and remain constant during the rest of the year. This indicated that the mussels were likely to be more sensitive to oxidative stress during gonadal development and gametogenesis in autumn and winter. In this period, mussels invested all their energy in reproduction. Also, such physiological state may have led to the lower levels of LMS and higher destabilisation of the lysosome membranes. It remains unclear to what extent the biomarker baseline levels in the present paper were responding to the reproductive state of the mussels and to environmental factors (water temperature and food availability),

or a combination of both. For example, the study on *M. edulis* from the Barents Sea reported that the lower the water temperature, the more destabilized were the membranes of lysosomes in haemocytes of the mussels (Camus et al., 2000). The reduction in LMS in *Mytilus* spp. were later explained by an increased physiological stress due to low food availability and intense energy demand of gonadal development during winter time (Hagger et al., 2010; Nahrgang et al., 2013; Balbi et al., 2017). Spawning in Rakkfjorden happened at the same time when water temperature was increased and phytoplankton as a food source for mussels was abundant. In the multivariate statistical analyses used in the present paper, all environmental factors resulted in the poor models and consequently were found to be difficult to use for explaining ecological aspects behind biomarkers and what they really responded to.

Tidal zone as a factor strongly influencing the biomarker baseline levels of the mussels from Rakkfjorden was also reported in the results of the multivariate statistics run for the present data. Indeed, the differences in physical conditions of the tidal zones within the mussel bed (e.g. wave energy, sand particle size, desiccation, light, temperature) are known to affect morphology of the mussel shell (McLachlan et al., 1995) and physiology of the mussels (Altieri, 2006; Petes et al., 2008; Tagliarolo et al., 2012). The air and water temperature logs in Rakkfjorden over the year indicated that mussels in the upper littoral zone were exposed to higher variations of the air temperature (from −10 to +16 °C) than mussels at the lower littoral zone that were in water most of the time (from 0 to +12 °C). Also, the access to food was reduced on the upper littoral zone due to tidal range. Due to natural differences in temperature, time to air exposure and food availability between upper and lower littoral zones, mussels inhabiting them were different in size and CI. The largest mussels in the lower littoral zone had higher volume and higher CI than the largest individuals in the upper littoral zone. In the west coast of Ireland, the larger CI was also reported in *Mytilus* spp. from the low shore compared to the high shore, and this difference was mainly linked to a shorter feeding time on the high shore (Schmidt et al., 2012). It was also observed that shells of the mussels on the upper littoral zone were more rounded than in mussels on the lower littoral zone having the shape of an egg, or ovoid. Such morphological differences might be associated with adaptation of the mussels to keep water inside during low tides and protect their soft tissues from drying (Steffani and Branch, 2003). As for the physiological adaptations, mussels from the upper littoral zone seemed to spawn later than mussels from the lower littoral zone. These differences between tidal zones were possibly a result of the different degree of environmental stress experienced by the mussels. According to Petes et al. (2008), mussels in the upper littoral zone had to invest all their resources to face increased stress due to harsher environmental conditions, and thus were not able to invest as much energy on reproduction as mussels in the lower littoral zone.

Taking into account the morphological and physiological differences in mussels at the different tidal zones, the biomarker baseline levels in the mussels across tidal zones were expected to be different too. However, no differences in LMS were found between the tidal zones in Rakkfjorden. The obtained results seemed to be a first evidence of no significant change in LMS in haemocyte lysosomes between upper and lower littoral zone mussels, at least in the Arctic, although changes in LMS with tide have been shown in digestive cells (Tremblay and Pellerin-Massicotte, 1997; Izagirre et al., 2008; Brenner et al., 2014). Indeed, the LMS was found to be reduced in the subtidal mussels than in intertidal mussels from the German bight of the North Sea in spring, because subtidal mussels reached maturity during winter and spring consuming higher energy for the reproductive process, whereas intertidal mussels stayed at the resting stage the whole winter and rapidly developed their gonads in spring in order to be able to spawn in summer (Brenner et al., 2014). Also, the present data indicated only few sporadic differences in the biomarkers for antioxidant defense between the tidal zones. GST activity was higher in mussels from the upper littoral zone than from the lower littoral zone in January, suggesting a need for increased antioxidant protection in more stressful habitats of the upper littoral zone (Letendre et al., 2009; Schmidt et al., 2012). Indeed, the difference between water and air temperature was highest in January, the coldest month in the studied year (Fig. S3). However, in contrast to higher CAT activity (Letendre et al., 2009) and higher TBARS levels (Schmidt et al., 2012) on the high shore compared to the low shore, the mussels from Rakkfjorden had higher CAT activity in lower littoral zone than in the upper littoral zone in November, while TBARS levels were similar in both tidal zones.

Even though the present study obtained quite detailed seasonal data on both abiotic and biotic factors that might have influenced the biomarker baseline levels of the mussels, the interpretation of the overall results was challenging. The number of collected samples (n = 9–12 per month from each tidal zone, Table S1) seemed not to be optimal as the individuals within the same sample group varied in terms of gender and reproduction stage. The number of mussels per tidal zone per sampling month could be increased to 20–30 to minimise this variation among individuals, and to increase n for statistical analyses.

A certain variance in the biomarker baseline levels could also possibly be due to different species or hybrids (Brooks et al., 2015). In fact, a recent study by Mathiesen et al. (2016) of the same mussel population as in the present study, showed the presence of *M. edulis* (70%), *M. galloprovincialis* (10%) and hybrids (20%). As it is not known to which degree the measured baseline responses may be affected across mussel populations and among species and hybrids, the use of genetic analysis with DNA barcoding may be important to implement in future studies.

The collected mussels were smaller in size than the mussels from the same location that were studied by Nahrgang et al. (2013). Indeed, the mean (±SE) length of the mussels in the upper littoral zone was 34.0 ± 0.3 mm and in the lower littoral zone it was 40.9 ± 0.6 mm (Table S2), while the length of the mussels reported in Nahrgang et al. (2013) ranged between 45 and 51 mm. Thus, the soft tissues especially after spawning period and during the winter time were not always enough to carry out all the biomarker analyses. This resulted in many gaps in the dataset and consequently in poor statistical models.

Concerning the assessment of LMS in living haemocytes using the neutral red retention assay (Martínez-Gómez et al., 2015), the validity of the seasonal results showing the reduction in LMS in *Mytilus* spp. in the winter half-year (Fig. 3) may be questioned. Indeed, the assay, that followed standardized protocols of ICES (Martínez-Gómez et al., 2015) was run in an ice-water cooled chamber placed in the laboratory (ambient air temperature around 22 °C throughout the year). The average air temperature inside the chamber was 17.7 ± 0.3 °C while performing all the assays. However, both water and air temperatures of the mussel environment varied significantly during the year. Mussels are ectothermic animals with ambient temperature being the most important factor influencing their physiological mechanisms and ecology (Huey and Stevenson, 1979; Angilletta et al., 2002). During the assay, the cells were exposed by fluctuating temperature both inside the chamber due to multiple door openings, and on the microscope slides that were taken out of the chamber for frequent evaluation under the light of the microscope, even when a slide check took no >10 s. Thus, the temperature conditions of the assay, due to fluctuations and levels far higher than ambient temperature, might invalidate the LMS data, especially in November, December and March, when the average LMS levels were the lowest. The haemocytes of the mussels collected in these months likely experienced large temperature jumps (from water temperature of 6, 3 and 3 °C, respectively, to at least 17.7 ± 0.3 °C inside the chamber) that could have caused a premature destabilisation of their membranes during the assay's conditions. In the experiments on thermal stress on *M. edulis*, the destabilisation of the lysosome membranes in the digestive cells were reported to be induced at 13 °C over ambient temperature (Moore, 1976). Hence, the neutral red retention assay protocol needs to be further refined. A

suggestions would be to evaluate the LMS at the mussels' ambient temperature by performing the assay in a climate room, adjusted to the ambient water temperatures at a given time. Otherwise, in order to reduce variability during the critical assessment phase, an alternative protocol for LMS in frozen tissue sections of digestive glands (e.g. enzyme cytochemical method; see more in Martínez-Gómez et al., 2015) may be considered. In contrast to the results obtained in the present study, measurement of the LMS in *M. galloprovincialis* from the northern coast of Spain by method for N-Acetyl-β-hexosaminidase indicated that lysosome membranes in the digestive cells were more destabilized in summer than in winter due to the higher feeding activity in summer (Izagirre et al., 2008). Also, LMS in these lysosomes was found to be dependent on the digestion and amount of time mussels were air exposed during the tidal drawdown, and not on the ambient temperature. Thus, the verification of the neutral red retention assay is highly needed, as it could influence the results of monitoring and false the interpretation of monitoring data.

4.2. Spatial study

The biomarker data obtained in the site of the seasonal study in Rakkfjorden was compared with five other sites within a 60-km radius (Fig. 1) in order to determine how applicable the data from the Rakkfjorden site was within the region. Two of the sites (Tromvik and Brensholmen) were similar to Rakkfjorden in terms of the environmental factors (water temperature, salinity and Chl A). The site in Buktalandet differed from Rakkfjorden only in reduced salinity. Two other sites (Simaviknes and Oldervik) were characterized by lower water temperature, lower salinity (Oldervik) and higher Chl A (Simaviknes) compared to Rakkfjorden. The individual parameters of the mussels (shell volume, sex ratio and distribution of the reproductive stages) were similar in Rakkfjorden and Buktalandet. Mussels from Tromvik, Brensholmen and Oldervik were larger in shell volume than mussels from Rakkfjorden. In addition, most of the mussels from Tromvik were females, while more males were presented in the samples from Rakkfjorden. Most of the mussels from Simaviknes were in the late maturity phase and seemed to spawn later compared to the mussels from all other sites that were either in the resting stage 0 or had just started gametogenesis (stage I).

The levels of bioaccumulated PAHs in the mussel soft tissues indicated that mussels at all sites were moderately (Class II–no toxic effects) polluted (Molvær et al., 1997). Concentrations of metals at all sites were either at moderate (Class II–no toxic effects) or at marked (Class III–chronic effects) levels. None of the mussels were severely (Class IV–acute toxic effects) or extremely (Class V–severe acute toxic effects) contaminated by pollutants. Thus, all sites of the spatial study likely represented a current environmental quality status in the region affected by increases in maritime traffic during the past decade (Dybedal et al., 2015; Borch et al., 2016).

The bioaccumulated concentrations of PAHs and metals in the soft tissues of mussels from Rakkfjorden increased from being insignificant (Class I – background levels of PAHs, Cd, Cu, Hg, Pb, Zn) and moderate (Class II – no toxic effects of As) in June 2010 (Nahrgang et al., 2013) to moderate (Class II – no toxic effects of PAHs, Hg, Pb) and marked (Class III – chronic effects of As, Cd, Cu, Zn) measured in the present study in July 2014. The concentrations of Cr and Ni, however, became lower during these four years, but they still fell into the moderate degree of pollution (Class II – no toxic effects). For now, it is difficult to conclude that such increase in bioaccumulated levels of PAHs and metals in mussels from Rakkfjorden was a result of the increased pollution in the region. It is also known that there could be yearly variations in bioaccumulation due to different intensity of the phytoplankton blooms and inter-annual shift of the gametogenesis period (Fattorini et al., 2008). In addition, seasonal variations in lipid content in the soft tissues of the mussels and changes in food quality and quantity were reported to have an effect on the bioaccumulation in mussels (Nahrgang et al., 2013). Thus, several inter-annual measurements of bioaccumulated levels should be done to verify whether there truly is an increasing trend, or whether this is within a normal range of annual and seasonal fluctuations.

The variations in the environmental factors (water temperature, salinity and Chl A concentration in water) across the sites, few sporadic differences in the individual parameters of mussels (size, sex ratio and distribution of the reproductive stages) and their different bioaccumulation levels of NPDs, Cd, Cr, Cu, Pb, Zn were not reflected in the baseline levels of biomarkers (TBARS, CAT and GST; Fig. 8). The lack of differences in biomarker responsiveness to oxidative stress and pollutant levels measured in mussels between Rakkfjorden and five sites within a 60-km radius from it suggested that mussels from Rakkfjorden could be representative for mussel populations on the North Norwegian coast, or at least within the Tromsø region.

5. Conclusion

The design of the present study was based on the ICES guidelines (ICES, 2010) and practical recommendations given for the biomonitoring of the Arctic *Mytilus* spp. in Nahrgang et al. (2013). The present study examined baseline levels of commonly used pollution biomarkers in *Mytilus* spp. over the course of a year and indicated some exogenous and endogenous factors that could potentially affect these levels and provide a natural range of their variability. Moreover, mapping spatial baseline variations in biomarkers of the mussel populations from different tidal zones and different sampling sites within the Tromsø region on the North Norwegian coast contributed to the improvement of our ability to use mussels as sentinel species for environmental monitoring purposes in Arctic environments. The morphological, histological and biomarker data obtained for the studied mussels, as well as the logging of the environmental parameters, provided relevant baseline information and may be useful in future biomonitoring studies of the present region.

Combination of the data obtained in the present study and study by Nahrgang et al. (2013), as well as recommendations for future improvement of the present study in terms of optimal number of mussels per sampling, careful selection of the mussels large enough to carry out the biomarker analyses, measurement of their chemical bioaccumulation over several years and reassessment of the LMS adjusted to the ambient temperature of the mussels will definitely provide a thorough database for biomonitoring procedures in the Arctic regions.

Conflict of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.11.397.

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